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Characterization of Rat Liver-specific Methionine Adenosyltransferase Gene Promoter

ROLE OF DISTAL UPSTREAM *cis*-ACTING ELEMENTS IN THE REGULATION OF THE TRANSCRIPTIONAL ACTIVITY*

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Methionine adenosyltransferase is a ubiquitous enzyme that catalyzes the only known route of biosynthesis of *S*-adenosylmethionine, the major methyl group donor in cell metabolism. In mammals, two different methionine adenosyltransferases exist: one is confined to the liver, and the other one is distributed in extrahepatic tissues. In the present study, we report the cloning of the 5′-flanking region of liver-specific methionine adenosyltransferase gene from rat. Two closely spaced sites for transcriptional initiation were identified by primer extension analysis. The major transcription start site was determined to be 29 nucleotides downstream from the putative TATA box. Transient transfection assays of constructs containing sequentially deleted 5′-flanking sequences fused to the luciferase gene showed that rat hepatic methionine adenosyltransferase promoter was able to efficiently drive reporter expression not only in liver-type cells (rat hepatoma H35 cells and human hepatoblastoma HepG2 cells) but also in Chinese hamster ovary cells. Two regions spanning nucleotides −1251 to −958 and −197 to +65 were found to be crucial for the promoter efficiency. The distal upstream region contains elements that positively regulate promoter activity in H35 and HepG2 cells but are ineffective in Chinese hamster ovary cells. Eight protein binding sites were characterized in both regions by DNase I footprinting analysis. Two of these elements, sites A and B, located in the distal region, were found to be essential for the regulation of promoter activity. Electrophoretic mobility shift assays and competition experiments showed that site A is recognized by an NF1 protein. Site B was able to interact with a member of HNF-3 family when nuclear extracts from rat liver and H35 cells were used in the *in vitro* assay, but an additional binding activity to an NHF1-like protein was obtained with the hepatoma cell extracts. It is suggested that this differential binding can contribute to the cell specificity of promoter function.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) X80270.

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S-Adenosylmethionine plays a central role in cellular metabolism, being the major methyl group donor in transmethylation reactions and the source of propylamine moieties for polyamine biosynthesis (1, 2). *S*-Adenosylmethionine is synthesized by transfer of the adenosyl moiety from ATP to the sulfur atom of methionine, in a reaction catalyzed by the enzyme methionine adenosyltransferase (MAT[1]; ATP:L-methionine *S*-adenosyltransferase, EC 2.5.1.6) (3). The occurrence of MAT has been extensively studied in a variety of organisms, where different isoenzyme forms have been characterized (2). In mammals, biochemical and molecular cloning studies have revealed the existence of at least two MAT (for a consensus nomenclature for mammalian methionine adenosyltransferase genes and gene products, see Ref. 4). One is selectively expressed in the liver and the other one is distributed in non-hepatic tissues (reviewed in Ref. 5). The presence of a liver-specific isoenzyme is related to the main role of this organ in methionine metabolism. Thus, most of the methionine taken up from the diet is metabolized in the liver, and up to 85% of all transmethylation reactions occur in the liver (5).

Hepatic MAT is a cytosolic homo-oligomeric protein, found as a mixture of tetramers and dimers (6, 7). Its expression correlates well with liver growth and differentiation, having been proposed to be a marker of the differentiated state of the hepatocyte (8). There is growing evidence suggesting that this enzyme can be regulated at different levels by a variety of factors and under several pathological conditions. For instance, a serious decrease in the enzyme activity, without a concomitant reduction in the expression of the gene, has been found in several human hepatic disorders (9, 10), as well as in different experimental models of liver injury (11, 12). In contrast, a marked reduction of MAT gene expression has been reported in human hepatocarcinoma (13) and in a rat model of hypoxia-induced liver injury (14). On the other hand, glucocorticoids (15) and cAMP (8) increase the expression of the gene in rats, whereas insulin blocks the inducing effect of glucocorticoids (8). Altogether, these results suggest that hepatic MAT gene expression is regulated differently under various normal and pathophysiological conditions. The necessity of a strict regulation of the expression of this enzyme has been recently emphasized by the fact that a sustained enhancement in its synthesis is accompanied by a depletion of cellular ATP and NAD, and a greater sensitivity to oxidative cell injury (16).

To study molecular mechanisms underlying the regulation of hepatic MAT expression, we have isolated and characterized

[1] The abbreviations used are: MAT, ATP:L-methionine *S*-adenosyltransferase; bp, base pair(s); PCR, polymerase chain reaction; RSV, Rous sarcoma virus; HNF, hepatocyte nuclear factor; CHO, Chinese hamster ovary; kb, kilobase pairs.

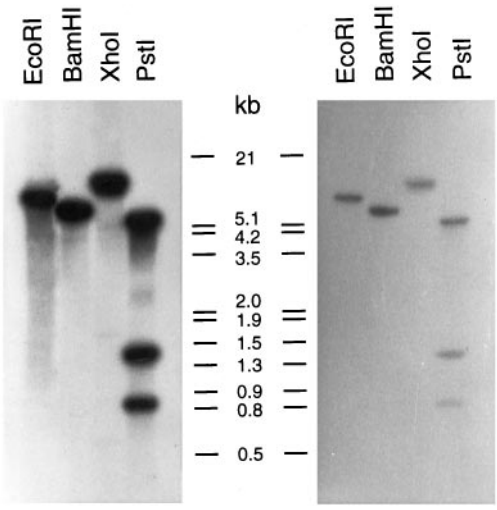

FIG. 1. **Southern blot analysis of rat genomic DNA.** High molecular mass genomic DNA from rat liver (10 μg per lane; *right panel*) and DNA from clone GRS4 (1 μg per lane; *left panel*) were digested with *Eco*RI, *Bam*HI, *Xho*I, or *Pst*I as indicated, separated on 1.2% agarose gel, and transferred to a nylon membrane. The radiolabeled probe was a 580-bp *Eco*RI/*Xba*I fragment of the rat liver MAT cDNA clone. λgt11 phage DNA digested with *Eco*RI and *Hin*dIII was used as a size marker; molecular sizes are indicated.

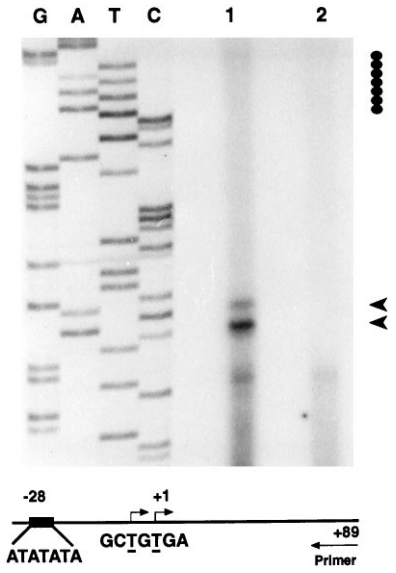

FIG. 2. **Determination of the transcription start site(s) of the rat liver MAT gene by primer extension.** A ^{32}P-end-labeled primer complementary to nucleotides −186/−163 of the rat liver MAT cDNA was annealed to rat liver poly(A)$^+$ RNA (*lane 1*) or yeast tRNA (*lane 2*) and extended with reverse transcriptase. The primer extended products were analyzed on 6% denaturing polyacrylamide gels, in parallel with sequencing reactions (*GATC*) carried out on the genomic subclone using the same primer. *Arrowheads* show the position of the extended products. *Solid circles* indicate the position of the putative TATA box. The sequences of the sense strand near the bands and the TATA box are shown *below*.

the 5′-flanking region of the rat MAT gene. The regulatory elements necessary for basal expression have been identified using transient transfections into various cell lines with hepatic MAT promoter-luciferase chimeric genes as well as by *in vitro* DNase I footprinting analysis. These *cis*-acting elements are clustered in a promoter proximal region and in a distal region. We finally show that transcriptional activity of hepatic MAT promoter is predominantly dependent on the binding of an NF1-like protein and liver-enriched transcription factors to the promoter distal region.

EXPERIMENTAL PROCEDURES

General Procedures—Standard procedures were used for screening the recombinant genomic library, restriction enzyme mapping, subcloning, DNA labeling, isolation of genomic DNA, Southern transfer, and hybridization to DNA on filters (17, 18). Oligonucleotide primers were synthesized on an Applied Biosystems 391 DNA synthesizer using the phosphoramidite method.

Isolation and Characterization of Genomic Clones—A rat genomic DNA library in EMBL-3 SP6/T7 (CLONTECH) was screened using as a probe a ^{32}P-labeled 580-bp *Eco*RI/*Xba*I fragment of the pSSRL cDNA clone (19), which contains 211 bp of the 5′-untranslated sequence and the first 369 bp of the rat liver MAT coding region. Five overlapping clones ranging in size between 13 and 18 kb kilobase pairs were isolated from ~1 × 10^6 recombinants. Clones were plaque-purified and subjected to Southern analysis. Fragments of interest were subcloned into the plasmid pUC18. DNA was sequenced on both strands by the dideoxy termination method (20), using Sequenase (U. S. Biochemical Corp.) and either pUC/M13 forward and reverse primers or sequence-specific oligonucleotide primers.

Primer Extension Analysis—Poly(A)$^+$ RNA used for primer extension studies was prepared from rat liver by oligo(dT)-cellulose chromatography (21). A 24-base oligonucleotide primer complementary to nucleotides −186/−163 of the rat liver MAT cDNA (19) was end-labeled with [γ-^{32}P]ATP using T_4 polynucleotide kinase. Three μg of rat liver poly(A)$^+$ RNA (or yeast tRNA as negative control) were annealed to 10^6 cpm of the primer and extended with 200 units of Moloney murine leukemia virus reverse transcriptase (Superscript II, Life Technologies, Inc.) as described previously (19). The primer-extended products were analyzed on 7 M urea, 6% polyacrylamide gels, in parallel with sequencing reactions carried out on the genomic subclone using the same primer.

Construction of Promoter-Luciferase Fusion Vectors—Eleven deletion constructs of different length were generated by PCR, using a MAT genomic clone as template. Initially, a DNA fragment spanning 1470 bp of the 5′-flanking region was amplified using 3′ and 5′ primers corresponding to nucleotides +49 to +65 and −1405 to −1389, respectively. The purified PCR product was inserted into the *Sma*I site of pUC18 plasmid. Ten deletions were then constructed by inverse PCR (22), using a vector-specific 5′ primer and 3′ primers complementary to nucleotides −1251 to −1235, −1154 to −1133, −1134 to −1118, −1080 to −1064, −958 to −942, −727 to −711, −527 to −511, −375 to −359, −193 to −177, and −87 to −71 of the cloned sequence. PCR reactions were performed as described previously (7) but using the thermostable DNA polymerase Dynazyme (Finnzymes Oy, Finland). The inserts of these 11 constructs were digested with *Kpn*I and *Sal*I and subcloned into the corresponding sites of the luciferase promoterless vector pXP1 (23). The identity of the constructs was confirmed by sequencing.

Transient Transfection Analysis—The relative transcriptional activities of MAT promoter fragments were determined by transient transfection analysis in cultured H35, HepG2, and CHO cells. Typically, cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and plated at approximately 3 × 10^5 cells/60-mm culture dish 24 h before transfections. The cells were transfected by the calcium phosphate precipitation method (18) with 6 μg (CHO) or 15 μg (H35, HepG2) of each DNA construct. Five μg of the β-galactosidase expression vector pCH110 (Pharmacia Biotech) were included as an internal standard of transfection efficiency.

After 18 h, the DNA precipitates were rinsed twice with phosphate-buffered saline, and cells were further grown for 24 h in culture medium. The cells were harvested in reporter lysis buffer (Promega), following the manufacturer's instructions, and the lysate was spun in a microcentrifuge for 15 s. Luciferase and β-galactosidase activities were determined as described (17, 18). All transfections were conducted in triplicate using at least two different batches of each construct. Activities reported are averaged from three independent experiments.

Preparation of Nuclear Extracts—Nuclear extracts from rat tissues or rat hepatoma H35 cells were prepared as described by Gorski *et al.* (24) and Andrews and Faller (25), respectively. The final nuclear suspension buffer contained 20 mM HEPES, pH 7.9, 0.4 M KCl, 0.2 mM EDTA, 1.5 mM $MgCl_2$, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, and 25% glycerol. Nuclear extracts were aliquoted and frozen at −70 °C. Protein concentrations were determined by the Bradford protein assay kit (Bio-Rad).

DNase I Footprinting Analysis—Two ^{32}P-end-labeled fragments of the 5′-flanking region of rat liver MAT gene were generated by PCR amplification of the template GSR4. The primers for these two frag-

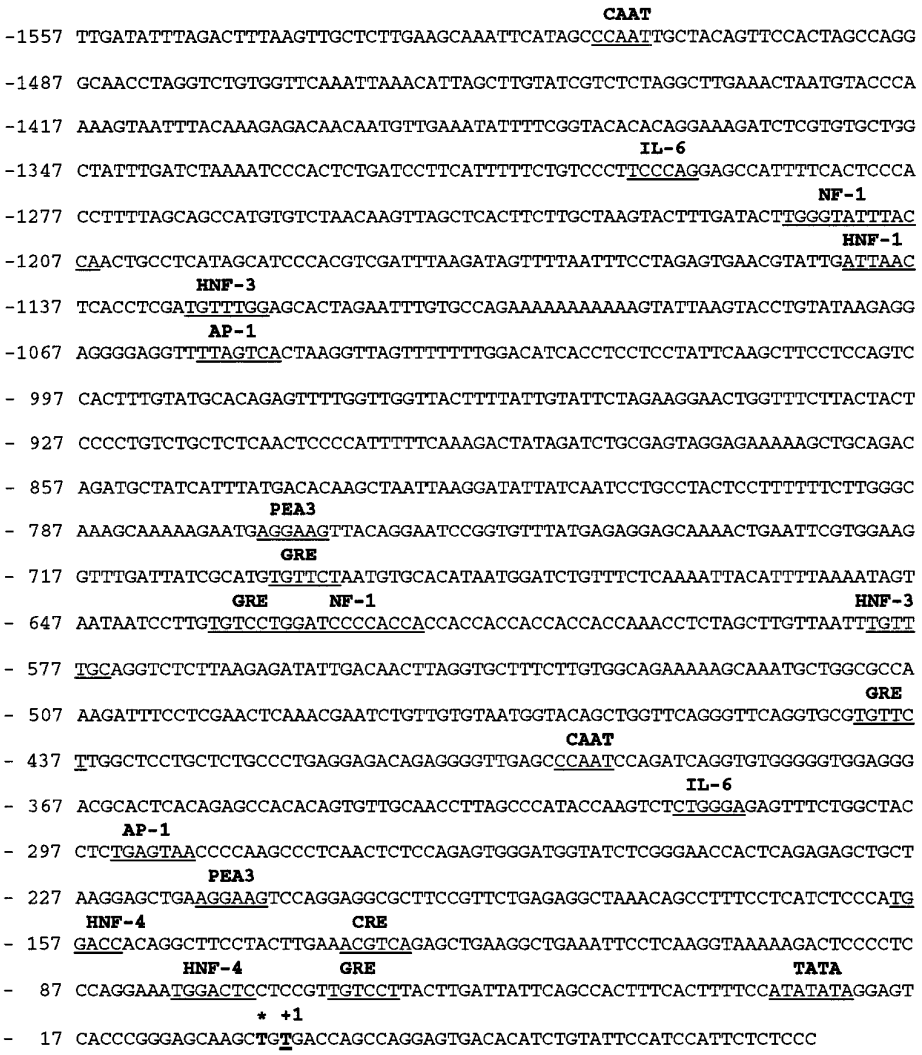

FIG. 3. **Nucleotide sequence and putative regulatory elements of the 5′-flanking region of rat liver MAT gene.** Sequence is numbered relative to the major transcription start site, which is designated as *+1*. The minor start site is marked with an *asterisk*. The putative regulatory elements are indicated in *bold letters* above the *underlined sequences*.

ments were +49 to +65, −193 to −177, −1012 to −996, and −1251 to −1235. One primer from each pair was 5′-end-labeled with [γ-^{32}P]ATP using T_4 polynucleotide kinase. PCR reactions were performed as described (7). Amplified fragments were purified using Sephacryl S-300 columns (Pharmacia). Approximately 5×10^4 cpm of end-labeled DNA fragments were incubated with 10–50 μg of nuclear proteins from rat liver. After 30 min incubation at room temperature, $CaCl_2$ and $MgCl_2$ were added to give a final concentration of 0.5 and 1 mM, respectively. DNase I digestions were performed at room temperature for 1 min, using different amounts of enzyme. The reactions were stopped by the addition of 140 μl of stop solution containing 2 μg of yeast tRNA, 20 mM EDTA, 150 mM sodium acetate, and 1.5 μg of proteinase K. Upon phenol extraction and ethanol precipitation, pellets were resuspended in sample dye buffer, and DNA fragments were resolved by electrophoresis in a denaturing 8% acrylamide/urea sequencing gel. The positions of specific DNase I-protected regions were determined by sequencing the fragments according to Maxam-Gilbert (26).

Electrophoretic Mobility Shift Assay—Electrophoretic mobility shift assays were performed in a 20-μl binding reaction containing 5 μg of crude nuclear extract, 40 mM HEPES, pH 7.5, 40 mM KCl, 0.2 mM EDTA, 5 mM $MgCl_2$, 1.25% Ficoll, 1 μg poly(dI-dC)·poly(dI-dC), 1 μg of sonicated herring sperm DNA, and, when indicated, a 50-fold molar excess of competitor oligonucleotide. The reaction mixtures were incubated on ice for 10 min, and then 6×10^4 cpm of annealed oligonucleotides were added, and incubation was continued for an additional 30 min. The DNA-protein complexes were resolved in a 6% acrylamide gel in 0.5 × TBE (1 × TBE: 89 mM Tris, pH 8.0, 89 mM boric acid, 2 mM EDTA). Double-stranded oligonucleotides used as probes or competitors were composed of the following sequences (top strand shown): MAT promoter FPA site, CACTAGAATTTGTGCCAGAAAAAAAAAAAGTA; FPB site, TGAACGTATTGATTAACTCACC; a mutant sequence (lowercase) of FPB site, oligonucleotide MtFPB, TGAACGcgTaGccTAACTCA; the HNF-1 binding site from the rat albumin promoter, TGTGGTTAATGATCTACAGT (27); the HNF-3 binding site from the transthyretin (TTR) promoter, GTTGACTAAGTCAATAATCAGAATCAG (28); a C/EBP binding site, GGTATGATTTTGTAATGGGGTA (29); a consensus NF-1 binding sequence, GCTTTGGCATGCTGCCAATATG (30); a consensus AP1 recognition site, ATTCTAGACTGAGTCATGGTACCGA (31).

RESULTS

Molecular Cloning of the 5′-Flanking Region of Rat Liver MAT Gene—A rat genomic DNA library in EMBL-3 sp6/T7 was screened with a 580-bp *Eco*RI/*Xba*I fragment of the rat liver MAT cDNA clone pSSRL, which comprises 211 bp of the 5′-untranslated sequence and the first 369 bp of the coding region (19). Five overlapping clones containing inserts ranging in size between 13 and 18 kb were isolated from approximately 1×10^6 recombinants. Restriction mapping and Southern analysis showed that all of them contained different lengths of the 5′-flanking region. A genomic clone of about 16 kb, designated GRS4, containing approximately 6 kb of the 5′-flanking region of the MAT gene was chosen for detailed analysis. To verify that no gross rearrangements had occurred during the cloning process, Southern blots prepared with rat genomic DNA digested with selected restriction endonucleases were hybridized with the same probe used for screening the genomic library. The sizes of the hybridizable fragments were identical to those obtained when Southern analysis of the genomic clone GRS4 was performed under the same conditions (Fig. 1). These re-

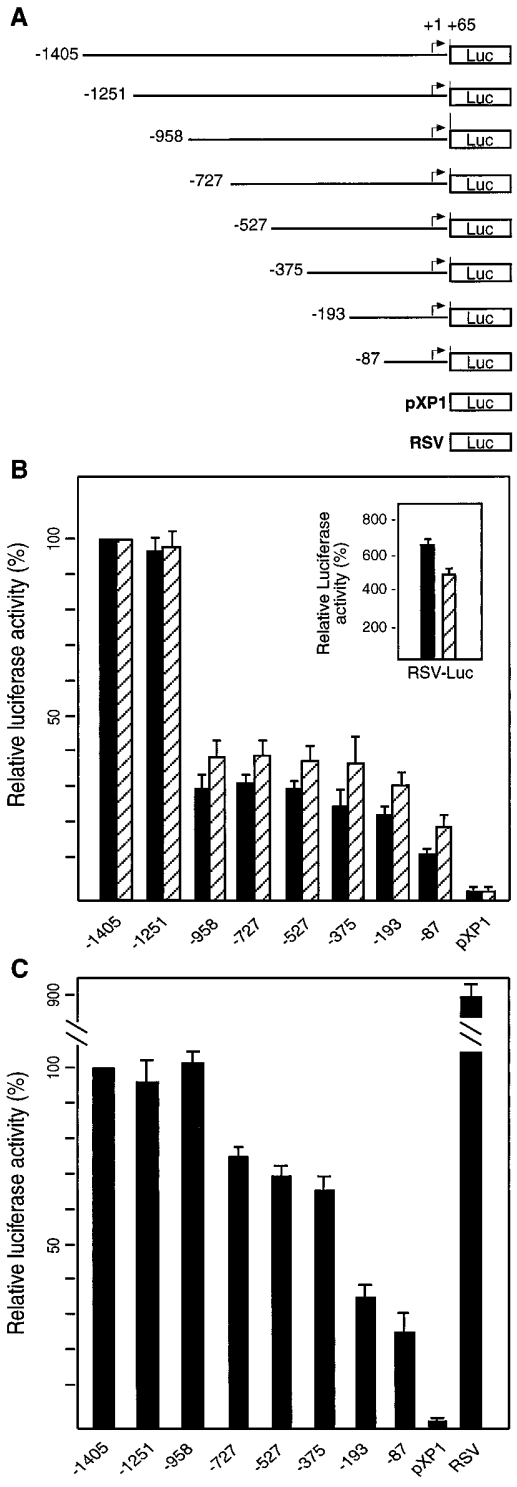

FIG. 4. **Transient transfection analyses of the rat liver MAT promoter/luciferase constructs.** *A*, schematic representation of the hepatic MAT-luciferase chimeric constructs. Progressive 5′ deletions of the MAT promoter extending from −1405 to +65 bp were generated by PCR (see "Experimental Procedures") and fused to the promoterless luciferase expression vector pXP1. Numbering is defined relative to the major transcriptional start site. *B* and *C*, graphical presentation of the transient luciferase activity in H35 (*filled bars*) and HepG2 cells (*hatched bars*) (*B*) and CHO (*C*) cells, transfected with the chimeric constructs described in *A*. Luciferase activities, normalized for β-galactosidase expression, are presented as a percentage of the activity of the −1405/+65 construct. The pXP1 vector without any promoter was used as negative control. Transfections with the positive control plasmid RSV-Luc were simultaneously performed (*inset* in *B*). The data represent the mean ± S.E. from three independent experiments performed in duplicate.

sults indicate that the cloned DNA segment retains the same sequence organization as in the genomic DNA and suggest that hepatic MAT gene is present as a single copy in the rat genome.

Determination of the Transcriptional Start Sites—To determine the start site of transcription, a primer extension assay was performed using poly(A)$^+$ RNA from rat liver, as described under "Experimental Procedures." As shown in Fig. 2, a major product corresponding to a 89-base extended fragment was detected as well as a minor product two nucleotides longer. These products were not detected when the assay was carried out using tRNA. Sequencing reactions performed on the genomic clone GRS4 using the same primer localized the major start site 251 nucleotides from the ATG translation initiation codon and 41 nucleotides upstream of the 5′-end of the partial cDNA sequence previously published (19). This base was designated as +1 for numbering the nucleotides in the gene.

Structural Features of the 5′-Flanking Region—The nucleotide sequence of rat hepatic MAT promoter region extending to 1557 bp upstream of the transcription initiation site is shown in Fig. 3. The exon sequence corresponding to the first 41 nucleotides of the MAT mRNA, not present in the reported cDNA sequence (19), is also included.

The 5′-flanking region shares 88% sequence identity with the 1113-bp promoter region reported for the mouse MAT gene (32). Sequence analysis revealed a putative TATA box located at positions −29 to −23, which is in agreement with the preferred position occupied by this element in a typical eukaryotic promoter (33). The canonical CAAT box, usually present around −80 bp, was not found in this area, but two perfect CAAT motifs were located far away (positions −379 and −1514). A number of DNA consensus sequences reported to bind specific trans-acting factors were also present. Thus, two AP-1 binding sites (34, 35) were found at positions −1057 to −1051 and −294 to −288. Two copies of the PEA3 motif (5′-AGGAAG-3′) (36), an oncogene-, growth factor-, and phorbol ester-responsive element, were located at −772 to −767 and −217 to −212. The consensus recognition motif for the glucocorticoid response element (TGT(C/T)CT) (37) was represented 4 times at positions −701 to −696, −636 to −631, −442 to −437, and −66 to −61. Two interleukin-6 response elements (38), which act as positive elements in several acute phase protein genes, were identified at −1301 to −1296, in reverse complement form, and at −317 to −312. Two NF-1 binding motifs (5′-TGGN$_7$CCA-3′) (39) were found at −1218 to −1206 and −630 to −618, the latter overlapping with a glucocorticoid response element. Finally, the analysis also revealed several DNA elements that matched the consensus binding sequences for liver-enriched transcription factors, such as HNF-1 (27) (at −1144 to −1138), HNF-3 (40) (at −1128 to 1122 and −581 to −575), and HNF-4 (41) (at −159 to −154 and −79 to −73).

Functional Analysis of the 5′-Flanking Region of Rat Liver MAT Gene—To delineate the sequences that drive MAT expression, a series of deletion mutants extending from −1405 to +65 bp was generated by PCR and cloned into the promoterless luciferase expression vector pXP1 (Fig. 4*A*). A similar plasmid containing the RSV promoter was used as a positive control for transfection. These chimeric constructs were transiently transfected into two cell types of liver origin, the rat hepatoma H35 cell line and the human hepatoblastoma HepG2 cell line, as well as into CHO cells. In all transfection experiments, a vector expressing β-galactosidase was included as internal control. Transient expression of luciferase activity showed that the MAT-luciferase vectors were expressed in the three cell types. The pattern of transcriptional activity of the chimeric constructs was very similar in H35 and HepG2 cells (Fig. 4*B*). Only background expression was observed following transfection

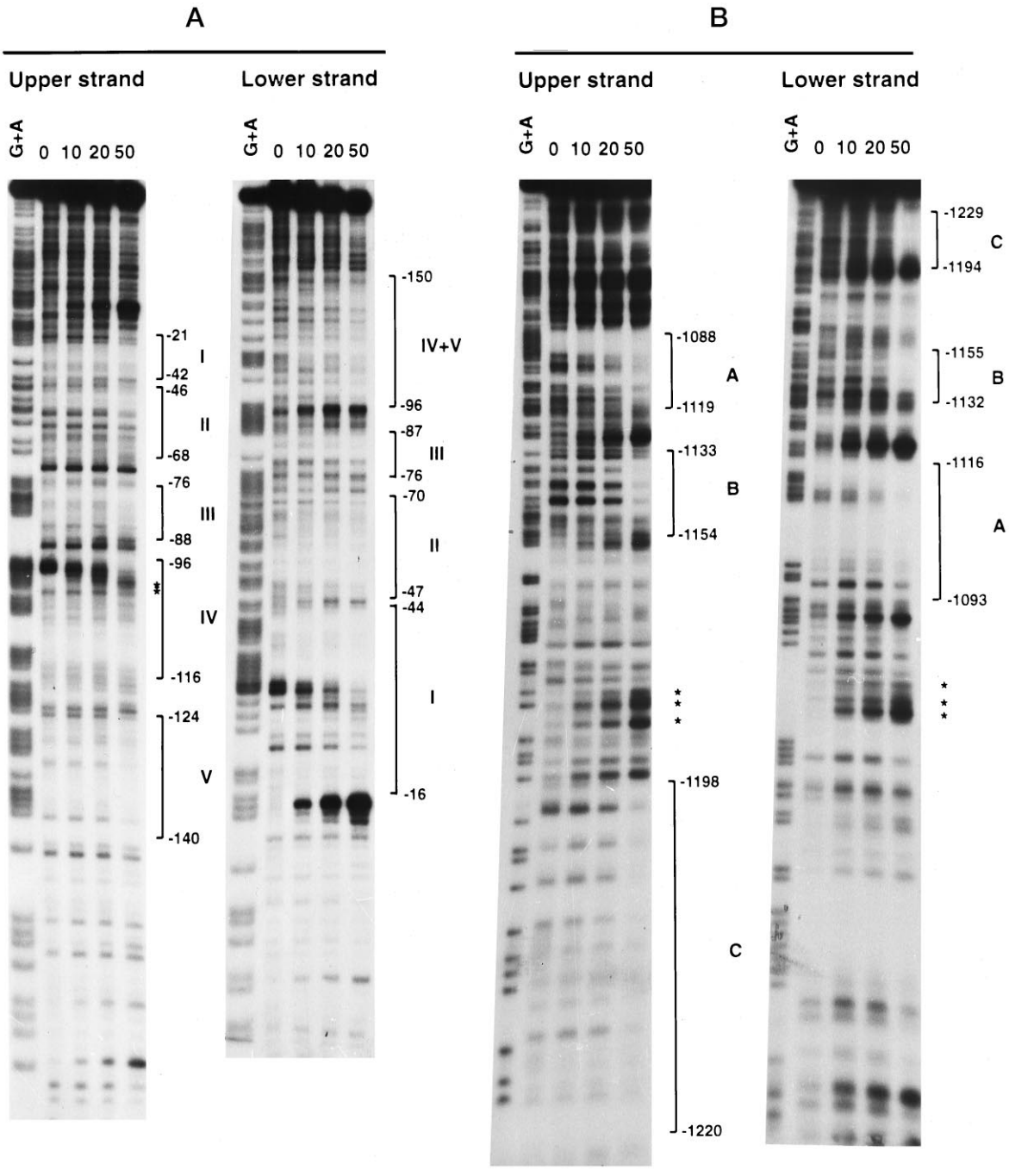

FIG. 5. **DNase I footprinting analysis of ^{32}P-labeled MAT 5′-flanking fragments −193 to +65 and −1251 to −996.** Double-stranded fragments corresponding to nucleotides −193 to +65 (*A*) and −1251 to −996 (*B*) of the 5′-flanking region of MAT gene were synthesized by PCR with 5′-^{32}P label on either strand and digested with different amounts of DNase I in the absence (*0*) or presence of *10*, *20*, and *50* μg of rat liver nuclear extract. Positions of protected regions are indicated by *brackets*, and major hypersensitive sites are marked with *asterisks*. *Lanes G+A* represent a Maxam-Gilbert sequencing reaction in the same fragments.

with the pXP1 luciferase vector without any promoter fragment. The highest luciferase activity, almost 14 and 20% of RSV-luciferase activity in H35 and HepG2, respectively, was observed with the −1405/+65 construct. Removal of the fragment −1405 to −1251 had little effect on the promoter efficiency. However, successive 5′ deletions from −1251 to −87 bp resulted in a marked decrease in luciferase activity, indicating the presence of a positive-acting region between −1251 and −958, that increases transcriptional activity about 3-fold. Deletions of sequences from −958 to −727, −727 to −527, −527 to −375, and −375 to −193 did not significantly affect luciferase activity, suggesting that no relevant additional elements involved in basal transcription are contained in this area. Further deletion from −193 to −87 promoted a 2-fold decrease, suggesting the presence of positive regulatory element(s) in this area. The resulting construct (−87/+65), which contains the minimal promoter elements including the TATA box, produced 10 and 18% maximal activity in H35 and HepG2 cells, respectively.

The profile of luciferase expression in CHO cells was quite different from that obtained in the liver-type cells (Fig. 4*C*). The constructs −1405/+65, −1251/+65, and −958/+65 yielded a similar luciferase activity. Therefore, as judged by the results obtained in the hepatic cells, the region comprised between

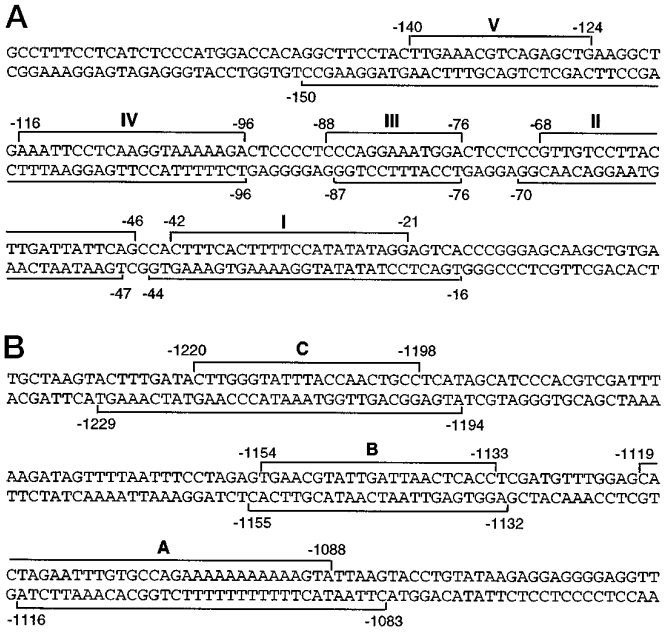

FIG. 6. **Nucleotide sequences protected from DNase I digestion.** Sequences protected against DNase I digestion by hepatic nuclear proteins are indicated by *bars above* (coding strand) and *below* (noncoding strand). *A* and *B* represent MAT promoter proximal and distal regions, respectively.

−1251 and −958 might contain tissue-specific regulatory elements that do not account for the promoter activity in CHO cells. On the other hand, and in contrast with the pattern observed in H35 and HepG2 cells, sequences from −958 to −727 and −375 to −195 seem to contain elements that positively regulate transcription in CHO cells.

DNase I Footprinting Analysis of MAT 5′-Flanking Region—From the results of deletion analysis, it can be concluded that the essential elements governing MAT expression in hepatic cells lie between nucleotides −1251 to −958 and −193 to +65. To identify these potential *cis*-acting elements, DNase I footprinting analysis was performed using both DNA fragments and nuclear proteins isolated from rat liver. As shown in Fig. 5*A*, five DNase I-protected areas, designated I to V, were generated on both strands within the promoter proximal region (−193 to +65) at increasing amounts of protein. The nucleotide sequences of these footprints are depicted in Fig. 6. Footprint I is a large protected area that covers putative TATA box elements. The second protected region extends from −46 to −70 and includes a significant homology to an AP1 recognition site. Footprint III shows no sequence similarity to any of the known protein-binding sites, as revealed by a search in transcription factor site data bases (42, 43). The region between −150 and −96 (footprints IV and V) adjoins sequences bearing some resemblance to several putative transcription factor binding sites, including cAMP response element, AP1, and AP4. Interestingly, this region also contains two tandem copies of the 5′-GTCGAA-3′ motif, which does not match any consensus recognition site for known transcription factors.

Three prominent DNase I-protected areas and several hypersensitive sites were identified upon incubation of a DNA fragment extending the promoter distal region from −1251 to −996 with liver nuclear extracts (Fig. 5*B*). Footprint A spans a putative NF1 binding site, and footprint B (−1132 to −1155) includes both HNF1 and HNF3 consensus sites homologies. Footprint C presents some sequence similarity to NF1 and MYOD recognition sites. Several DNase I-hypersensitive bands were also detected in this promoter region, even at low protein input (indicated with *asterisks* in Fig. 5*B*). These could also reflect the binding of nuclear proteins, which would make this area more accessible to the action of the DNase I.

To approach the identification of elements that could represent binding sites for liver-enriched transcription factors, DNase I footprinting assay was also performed using nuclear extracts from other rat tissues. As shown in Fig. 7*A*, incubation of the promoter proximal region with nuclear proteins from liver, kidney, or lung yielded similar DNase I footprints, showing a minimal variation in DNase I-hypersensitive cleavage accompanying footprint IV. It is therefore suggested that ubiquitous trans-acting factors are interacting with this promoter area. The promoter distal region exhibited a distinct DNase I protection pattern upon incubation with the nuclear extracts from different sources. Thus, while footprint A was detectable with all nuclear extracts, elements B and C were clearly protected only by nuclear proteins from liver, although the former seemed to be weakly protected by kidney extract (Fig. 7*B*). These findings suggest that element A represents a binding site for a ubiquitous transcription factor, whereas elements B and C interact with liver-enriched factors.

Relative Contribution of Protein-binding Sites in the Distal Promoter Region to Transcription Activity—Since the region spanning nucleotides −1251 to −958 appeared to be crucial for the promoter activity in hepatic cells, we next focused on the analysis of the functional significance of the protein binding sites detected by footprinting in this area. For this purpose, 5′ deletion mutants extending up to each footprint were carried out; the constructs were transfected in H35 and CHO cells, and the transcription capacity was assessed (Fig. 8). Removal of the fragment −1251 to −1154 containing the area corresponding to footprint C promoted a small decrease (about 20%) of luciferase activity. However, an additional deletion of sequences corresponding to element B (−1154 to −1134) resulted in about a 2-fold increase in reporter gene transcription, indicating the presence of elements that negatively influence the promoter function. Finally, upon deleting a fragment comprising also the footprint A, the promoter efficiency was reduced 3-fold, rendering a relative luciferase activity similar to that exhibited by the deletion construct −958/+65 (Fig. 4). On the other hand, and in agreement with the data shown in Fig. 4, no differences in luciferase activity were detected upon transfection of these deletion constructs in CHO cells.

Characterization of Nuclear Protein Binding to Sequences within Footprints A and B—Since sequences residing within footprints A and B appeared to be of major functional relevance, the identity of the potential factors that interact with these elements was explored by gel mobility shift analysis. Double-stranded synthetic oligonucleotides (named FPA and FPB; see "Experimental Procedures") encompassing each of the two DNase I footprinted sites were used as probes and incubated with nuclear extracts from rat liver and H35 cells. To assess the specificity of each protein binding site, a series of double-stranded oligonucleotides was used as competitors in the binding reactions.

As shown in Fig. 9, the radiolabeled FPA probe formed two closely spaced DNA-protein complexes with rat liver and H35 nuclear extracts. The specificity of DNA binding was established by the ability of a 50-fold molar excess of the same unlabeled probe to compete for complex formation. An NF1 consensus oligonucleotide efficiently competed, even better than the homologous FPA, for binding with both nuclear extracts, whereas no competition was observed with oligonucleotides containing HNF-1, HNF-3, C/EBP, or AP1 consensus sequences. This result suggests that the protein forming these complexes is a member of the NF1 family or an NF1-like protein.

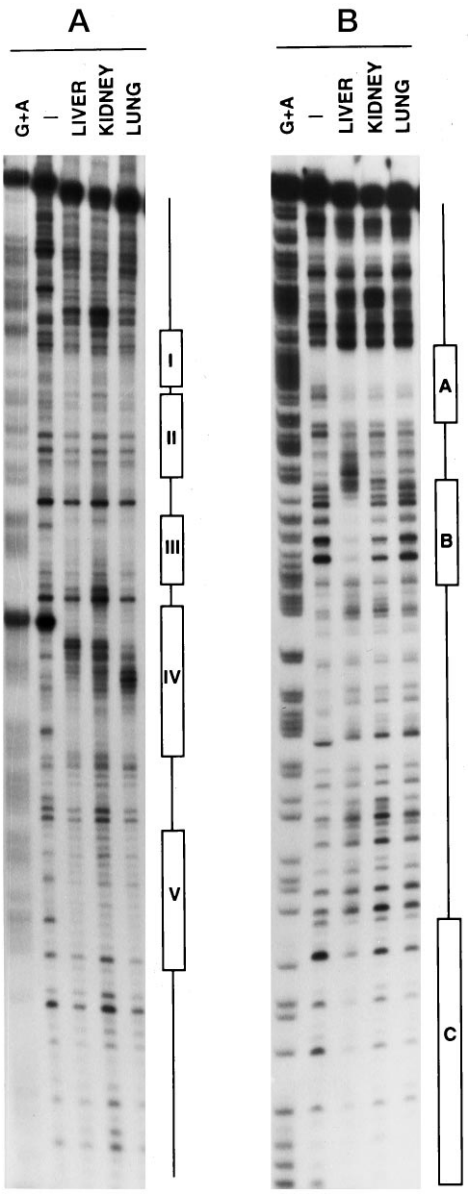

FIG. 7. **DNase I footprinting analysis of MAT promoter using liver and non-liver nuclear extracts.** DNA fragments comprising nucleotides −193 to +65 (*A*) and −1251 to −996 (*B*) of the 5′-flanking region of MAT gene were incubated with 50 μg of nuclear extracts from the indicated tissues. Probes were labeled on the upper strand. After DNase I digestion, samples were resolved on sequencing gels. *Lane* −, no extract added; *lane G+A*, Maxam-Gilbert reaction. Protected regions are *boxed*.

Footprint B displays sequence homology to consensus binding sites for the liver-enriched factors HNF-1 and HNF-3. Accordingly, competition experiments were carried out using unlabeled oligonucleotides corresponding to binding sequences for these factors, as well as for C/EBP. Two major gel shift complexes were detected when the FPB probe was incubated with nuclear extracts from rat liver; the upper one fully competed by a 50-fold molar excess of the unlabeled oligonucleotide (Fig. 10). Addition of a mutant form of FPB sequence (MtFPB) or a C/EBP consensus element failed to compete with protein binding. However, an HNF-3 oligonucleotide markedly reduced the complex formation. This was also competed, although to a lesser extent, by an HNF-1 consensus binding site. Nuclear extracts from H35 cells formed a different pattern of shifted complexes when incubated with radiolabeled FPB. Thus, one complex of similar electrophoretic mobility to the major complex formed with rat liver extracts was detected, as well as an additional complex of slower mobility. Both shifted bands could be abolished by addition of excess unlabeled probe but not by the mutated FPB sequence or C/EBP consensus element. The formation of the upper complex was abolished by an excess of HNF-1 oligonucleotide, which also showed a weak cross-competition with the lower complex. Conversely, addition of an HNF-3 consensus sequence did not affect the formation of the upper complex but efficiently competed with the lower complex. In fact, the latter exhibited a similar behavior in competition experiments with both cell extracts. In summary, these results suggest that in both cell extracts MAT promoter FPB element is interacting with an HNF3-like protein, whereas in the H35 extracts, the same element also binds an HNF1-like factor.

DISCUSSION

Among mammalian tissues, the liver is a major site of production of MAT, needed to metabolize most of the methionine taken from the diet and to provide key metabolites for the cell. The crucial role of the liver-specific MAT (1, 5, 44, 45) requires a precise regulation of its expression under basal conditions and in response to different stimuli. In this study, we report the cloning and initial characterization of the 5′-flanking region of the rat hepatic MAT gene, providing insights into the mechanisms that regulate its expression.

The gene for the rat liver-specific MAT is present as a single copy in the genome, as has also been reported for its human (10) and mouse (32) counterparts. The major start site of transcription was determined to be 29 nucleotides downstream of the putative TATA sequence, at the same site as that for the mouse MAT gene (32), further supporting the assignation of this nucleotide as the cap site. However, unlike the mouse gene, this site corresponds to a T residue, a nucleotide not usually found at the transcription start site in the eukaryotic promoters analyzed (33).

Functional analysis by transient transfections has shown that the promoter for the rat hepatic MAT is active not only in liver-type cells but also in a non-hepatic cell such as CHO. This finding raises the possibility that the liver-restricted expression of the endogenous gene is not mediated by the action of tissue-specific transcription factors. In such cases, the lack of its expression in the extrahepatic tissues could be due to situations known to stably repress the transcription of many tissue-specific genes, such as DNA methylation or genome organization (*i.e.* compaction of the chromatin) (46, 47). By analyzing a series of 5′-end deletions, two major regulatory regions have been identified, a promoter proximal region (−193 bp relative to the transcription start site) and a far upstream area extending nucleotides −1251 to −958. Most notably, promoter elements located in the latter produced the highest reporter activity upon transfection in H35 or HepG2 cells.

It is interesting to note that major differences can be found when the functional analysis of the 5′-flanking region of rat liver MAT gene is compared with that reported for its mouse counterpart, despite the high sequence conservation in the 1113-bp overlapping regions. Basically, in the mouse promoter, the 5′ *cis*-acting elements that produce the highest transcriptional activity are located between nucleotides −365 to −145, and a negatively acting region has been identified between −518 to −366 (32). Nevertheless, it is not known whether the crucial distal elements found in the rat promoter are also present in its mouse counterpart, since in the latter this region has not been analyzed.

The promoter proximal region of rat hepatic MAT appears to contain positive elements commonly active in both hepatic-type and CHO cells. Thus, the region spanning positions −87 to +65 exhibited similar relative transcriptional activity in the three

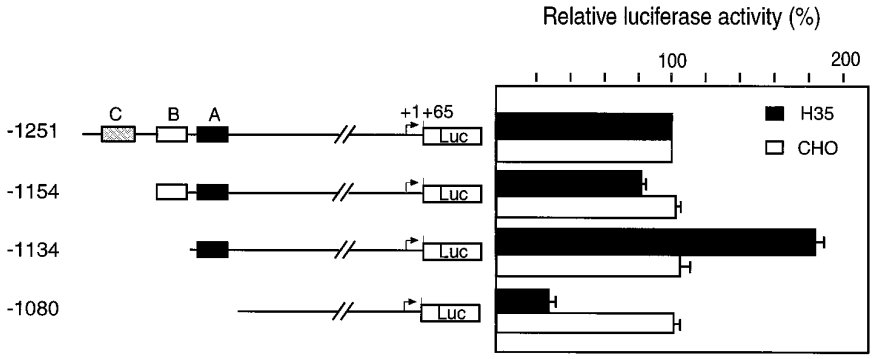

FIG. 8. **Role of distal *cis*-acting elements in the transcriptional activity of hepatic MAT promoter.** Rat hepatoma H35 and CHO cells were transiently transfected with the indicated MAT promoter-luciferase constructs. Luciferase activities are expressed as a percentage of the activity of the −1251/+65 construct. Data are the mean ± S.E. from three independent experiments performed in duplicate. *Boxes* represent the three DNase I-footprinted regions.

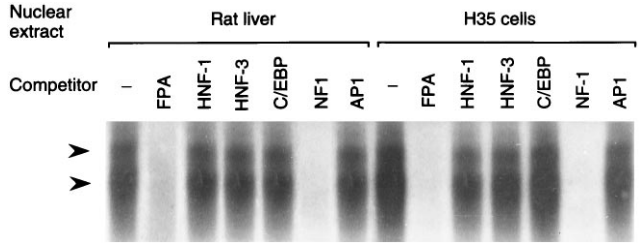

FIG. 9. **Gel mobility shift assay of nuclear proteins interacting with the FPA sequence proteins binding to the MAT promoter FPA sequence.** Labeled oligonucleotide probe corresponding to DNase I-footprinted region A was incubated with 5 μg of nuclear extracts from rat liver or rat hepatoma H35 cells. For specific competition, a 50-fold molar excess of unlabeled oligonucleotide corresponding to binding sites for HNF-1, HNF-3, c/EBP, NF1, and AP1 were used (see "Experimental Procedures" for the sequences of the oligonucleotides). The binding reactions were separated by electrophoresis in a native 6% polyacrylamide gel. DNA-protein complexes are indicated by *arrowheads*.

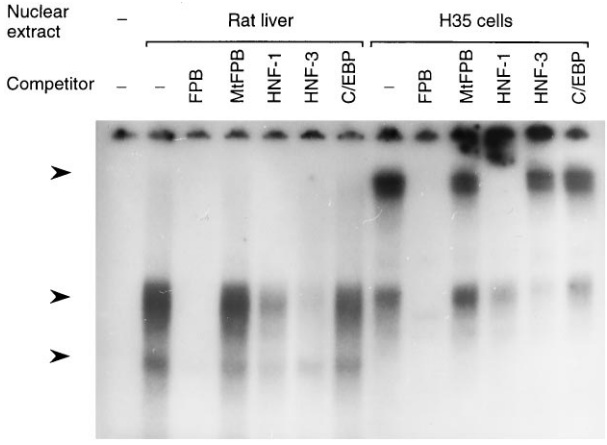

FIG. 10. **Differential binding of nuclear proteins from rat liver and H35 cells to the FPB sequence.** Gel shift analysis of the interactions of FPA with rat liver and H35 nuclear proteins. Competition was carried out with a 50-fold molar excess of the indicated unlabeled oligonucleotides. An FPB mutant (MtFPB; see "Experimental Procedures") was included as competitor. *Arrowheads* indicate the specific binding complexes.

cell lines tested, probably owing to the action of basic regulatory sequences such as TATA box. In fact, three protected elements, one of them covering the putative TATA box, were detected in this DNA fragment by using the *in vitro* footprinting assay, and they appear to represent binding sites for ubiquitous trans-acting factors, as judged by analysis with nuclear extracts from different sources. Two additional footprinted areas were found between nucleotides −193 and −87. These elements also seem to be of functional relevance since its deletion produced a significant decrease in promoter activity. The identity of the proteins binding to this proximal region is the subject of further investigation.

The distal region is crucial for basal and tissue-specific promoter activity. Thus, its removal led to a drastic reduction in the reporter activity upon transfection in H35 and HepG2 cells but had no effect in CHO cells. The structure-function relationship of this area was studied by a combination of DNase I footprinting analysis, transient transfections, and gel shift assays. With this approach, three relevant *cis*-acting elements (A–C) were defined. Sequences spanning sites A and C bind factors that positively regulate transcription of MAT promoter, with a predominant role for site A. Gel retardation and competition experiments have provided evidence that this element interacts with a member of the NF1 family, a finding that is consistent with the initial identification by sequence homologies. Our results suggest that NF1, which usually acts as a positive transcription factor (30, 48), plays a key role in directing MAT expression.

On the other hand, site B appears to bind a factor that negatively modulates transcriptional activity in H35 cells. Surprisingly, an oligonucleotide containing this element showed, in gel shift assays, different specific interactions with nuclear extracts from rat liver and hepatoma H35 cells. Thus, it was able to recognize a protein present in both cell extracts that, as judged by competition experiments, appears to be a member of the liver-enriched transcription factor HNF-3 family. However, an additional binding activity, corresponding to a potential HNF-1 transcription factor, was detected with the H35 extract. It is interesting to note that site B contains the sequence 5′-CGTATTGATTAAC-3′, which is 90 and 84% identical to the consensus HNF-3 (49) and HNF-1 (27) binding elements, respectively. Therefore, both factors could be partially interacting with the same core sequence. This assumption is supported by the fact that a mutated form of element B disrupting this sequence was unable to compete with the wild-type probe for the formation of both protein complexes. The absence of HNF-1 binding activity in the liver extract is, however, difficult to explain, since hepatic tissue expresses high levels of this transcription factor (50, 51). One possible explanation derives from the fact that de-differentiated hepatoma cells do not express HNF-1 but present a protein called variant HNF-1 (vHNF-1 or HNF1β, Refs. 52 and 53). Although both factors can interact with the same target elements, it has been suggested that the sequence of the binding site may modulate the affinities of HNF-1 proteins, thus providing another level of regulation (54). Therefore, vHNF-1 or a related protein present in hepatoma cells might somehow display a higher affinity for element

B than HNF-1 and then compete with HNF-3 binding. The finding that element B negatively regulates MAT transcription is in agreement with this hypothesis, since HNF1 is a positive regulatory factor (50, 51), and vHNF-1 has been shown to be involved in the negative regulation of some liver-specific genes (55). In this context, it should be mentioned that steady-state levels of liver-specific MAT mRNA are lower in rat hepatoma H35 cells than in hepatocytes (15). A similar pattern of reduced expression has been also detected for the human hepatic mRNA in the hepatoma HepG2 cell line,[2] a finding that does not completely agree with a previous report suggesting a complete lack of transcription of the liver MAT gene in these cells (13).

It is well documented that the successful expression of several liver-specific genes requires a combined action of ubiquitous factors, such as NF-1, and liver-enriched factors (50, 51, 56, 57). HNF-3 proteins function as transcriptional activators (58), and their cooperative interaction with NF-1 has been reported to be needed, among others, for the hepatocyte-specific expression of albumin gene (59) or for full activity of the promoter of C4b binding protein (60). A combined action of both factors might also account for the proper expression of rat liver MAT. In fact, their respective sequence elements are tandemly arrayed and separated by only 16 nucleotides. Therefore, the finding that in hepatoma cells an additional protein can interact with the HNF-3 element of MAT promoter raises the possibility that such binding could interfere with the transactivation ability of this transcription factor. In this line, the results presented here could explain the different basal expression of MAT in rat liver and hepatoma cells and provide more insights into the mechanisms governing the expression of liver-specific genes in differentiation.

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Characterization of Rat Liver-specific Methionine Adenosyltransferase Gene Promoter: ROLE OF DISTAL UPSTREAM cis-ACTING ELEMENTS IN THE REGULATION OF THE TRANSCRIPTIONAL ACTIVITY

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